THE ROLE OF *RAD51C* ON THE FANCONI ANEMIA PATHWAY IN ZEBRAFISH

by

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John Postlethwait

DNA is exposed to various forms of damage from both natural and anthropogenic causes. Normal cells repair this damage, which allows them to remain viable. However, cells with mutations in DNA repair genes are unable to repair the damage, which leads to cancers and/or cell death. My research examined *RAD51C*, a gene involved in DNA repair by homologous recombination. Homologous recombination repairs a form of DNA damage known as double strand breaks in which both strands of DNA are severed.

Mutations in *RAD51C* lead to breast and ovarian cancer in humans and, as of 2010, were thought to be related to Fanconi anemia (FA), but evidence was based on a single patient whose symptoms did not conform to the classical phenotype of FA. FA is a rare autosomal disorder characterized by chromosome instability, progressive bone marrow failure, developmental abnormalities, and a predisposition to cancer.

The goal of my research was to provide firm evidence whether mutations in *RAD51C* mimic FA symptoms. In zebrafish, FA involves female-to-male sex reversal, reduced fertility, and sensitivity to DNA crosslinking agents. I analyzed the effects of the *rad51c* mutation in zebrafish with respect to sex determination, fertility, early

hematopoietic gene expression, and the ability of cells to respond to DNA damage. My results showed that *rad51c* mutations in zebrafish do mimic the symptoms of bona fide zebrafish FA mutants and thus verify the designation of *rad51c* as an FA gene. This research will lead to a better understanding of the role of *rad51c* in human health, and will ultimately improve detection, prevention, and therapies for cancer and Fanconi anemia.

Acknowledgements

I would like to thank Professor John Postlethwait for the opportunity to conduct this research, it has been a pleasure working in your lab. I would also like to thank Catherine Wilson for being a mentor and helping hand throughout this process. Also, a special thanks to Trevor, Adam, Joe and the University of Oregon Fish Facility for their assistance and hard work in fish husbandry. I would also like to thank Kelly Sutherland of the Robert D. Clark Honors College for providing additional advice and perspective that has helped to improve this final product.

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Introduction

Throughout our life, our DNA is exposed to various forms of damage. This damage can take many forms, one of which is interstrand crosslinks (ICLs). These crosslinks are highly toxic DNA lesions that prevent transcription and replication by inhibiting DNA strand separation (Figure 1) [4]. ICLs can be caused by endogenous sources such as nitrous acid and aldehydes, or exogenous sources such as Cisplatin, a chemotherapy drug [4]. Another form of DNA damage is DNA double strand breaks (DSBs). These problems can be generated by genotoxic agents such as ionizing radiation and byproducts of cellular metabolism such as reactive oxygen species [4]. Additionally, DSBs are an intermediate step of ICL repair because to repair the crossover the DNA strands must be broken (Appendix 1) [4].

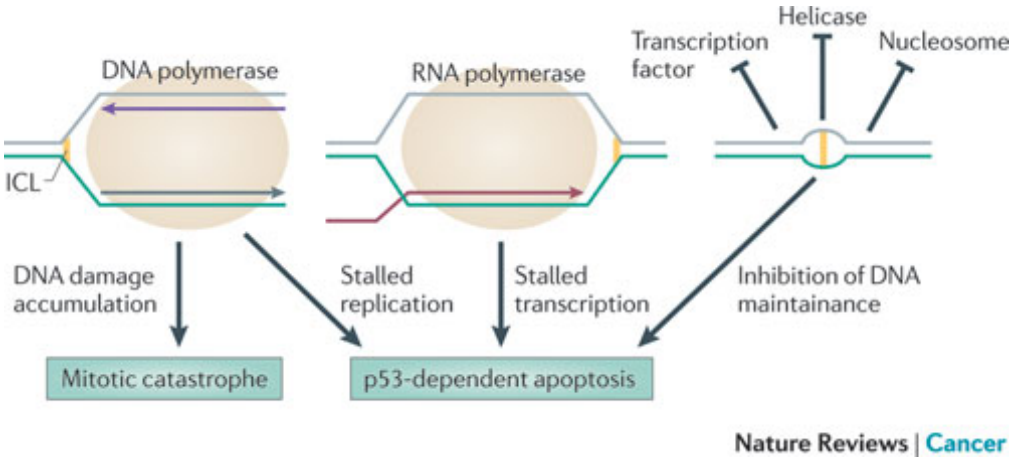


Figure 1: Examples of DNA crosslinking

DNA crosslinking interferes with both DNA replication and transcription and results in DNA damage, genome instability, and apoptosis (Deans and West, 2011, Nature Reviews, Cancer, 11: 467-480.).

These forms of DNA damage are dangerous for genomic stability but, thankfully, the body has various ways of repairing them. These damage repair processes are heavily

regulated and involve many proteins for them to function effectively. It is important that these repair processes work properly because insufficiency can lead to genome instability which enables the development of cancers [9].

One of the proteins involved in DNA repair is RAD51C. This protein is a part of a DNA repair pathway called the Fanconi anemia/BRCA (breast cancer) pathway. Bi-allelic mutations in *RAD51C* have been shown to cause a Fanconi anemia-like disorder, while heterozygous carriers have an elevated risk of breast and ovarian cancer. What follows is a discussion of the protein's role in the DNA repair response as well as how mutations in the protein affect the development of zebrafish.

Current Literature

Fanconi Anemia: Clinical and Cellular Features

Fanconi anemia (FA) is a rare autosomal or X-linked recessive disorder of chromosome instability [1,5,8,9,15,18]. FA effects anywhere from 1 in 200,000 to 1 in 400,000 live births [1]. There are currently 21 identified Fanconi anemia genes (FANC-A, B, C, D1 (BRCA2), D2, E, F, G, I, J, L, M, N (PALB2), O, P, Q, R (RAD51), S (BRCA1), T (UBE2T), U (XRCC2), and V (MAD2L2) [9,15]. The disease is highly heterogeneous and arises from bi-allelic mutations in at least one of these 21 genes [4,18].

Clinically, FA is characterized by progressive bone marrow failure, aplastic anemia, congenital abnormalities, and a predisposition to cancer [1,4,6,9, 18]. Some of the developmental abnormalities include microphthalmia, microcephaly, and hypoplastic radius and thumb (Figure 2) [17]. FA patients are particularly prone to developing acute myeloid leukemia as well as head and neck squamous cell carcinoma

[8,9,15,18]. Additionally, patients can develop abnormalities in the gonads, including hypogonadism, impaired gametogenesis, defective meiosis, and infertility [10]. The cells of FA patients exhibit chromosome instability, increased chromosomal aberrations, a defect in DNA interstrand crosslink-induced HR, and hypersensitivity to DNA interstand crosslinking agents (Figure 3) [1,4,5,8,9,15].

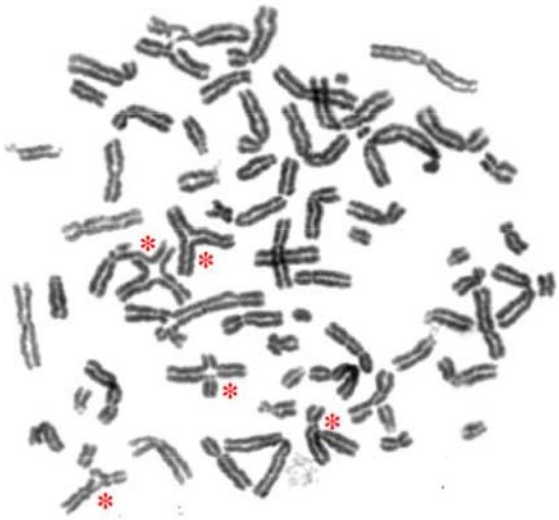


Figure 2 (left): Developmental abnormality caused by Fanconi Anemia

This x-ray shows a Fanconi anemia patient missing a thumb and the radius bone in the forearm (Image retrieved from https://radiopaedia.org/).

Figure 3 (right): Chromosomal aberrations of Fanconi patients

The red stars indicate atypical chromosomes (Moldovan and D'Andrea, 2009, Annual review of genetics, 43: 223-249.)

The Molecular Level of the Fanconi Anemia/BRCA Pathway

All of the FA genes are part of the FA/BRCA pathway. The FA/BRCA pathway is a critical tumor suppressive pathway that is involved in the repair of DNA double stand breaks [1,4,5,8,9,15]. As previously mentioned, DSBs are dangerous because they are a source of genome instability that can result in various cancers [9]. The FA/BRCA pathway is important in tumor suppression because it prevents cancer [9].

In the FA/BRCA pathway, eight of the FA genes (FANC-A -B, -C, -E, -F, -G, -L and -M) encode proteins that form what is known as the FA core complex (Figure 4) [5,17, 19]. This complex is recruited to ICLs via multiple mechanisms that are still not entirely known [19]. Once recruited to the site of DNA damage, the core complex triggers the monoubiquitination of FANCD2 and FANCI [5,16,17]. Monoubiquitinated FANCD2/FANCI then localizes at nuclear foci at FANCD1 [19]. Then, for reasons not fully understood, DNA repair proteins FANCD1 (BRCA2), BRCA1, and RAD51 come in and perform homologous recombination, repairing the lesion [5, 16, 19].

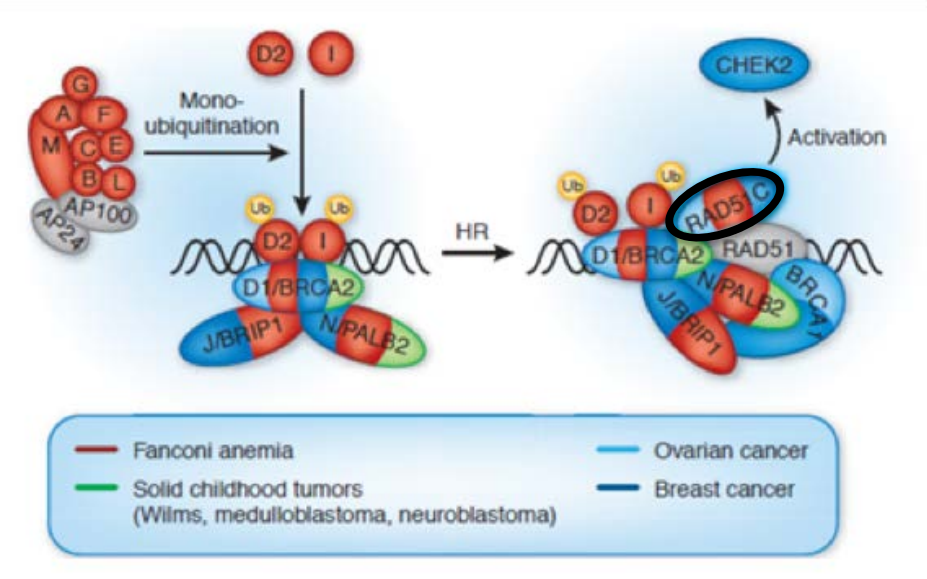


Figure 4: The Fanconi Anemia Pathway

The Fanconi anemia core complex (bundle of red proteins on the far left) comes in to the site of DNA damage and triggers the monoubiquitination of D2 and I. FancD2 and FancI then localize at FancD1/Brca2 causing other proteins, including Rad51c to come in and perform HR (Levy-Lahad, 2010, Nature genetics, 42.5: 368-370.).

The Role of RAD51C

RAD51C is a member of the RAD51 protein family which includes RAD51 and five paralogs in mammalian cells (RAD51B, RAD51C, RAD51D, XRCC2, and XRCC3) [3, 9,15]. The RAD51 proteins are important proteins involved in DNA damage repair in cells and during meiotic recombination [2]. They are important in carrying out an essential DNA damage repair mechanism known as homologous recombination (HR) [6,9, 18]. HR is a form of DNA repair in which nucleotide sequences are exchanged between two similar or identical strands of DNA (Appendix 1). It is the main form of DNA repair that mends DNA double strand breaks with high fidelity [8].

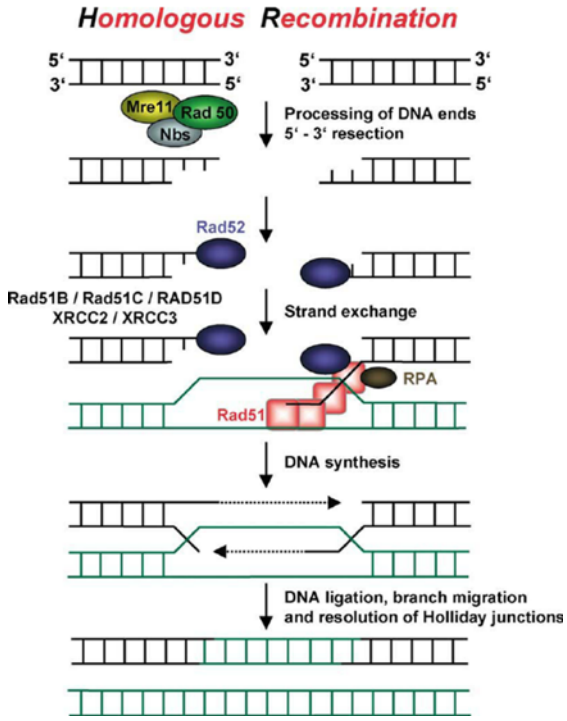


Figure 5: The role of RAD51C in homologous recombination

In homologous recombination, RAD51C plays an important role in recruiting RAD52 and initiating the strand inversion step (Christmann, Markus, et al., 2003, Toxicology, 193(1): 3-34.).

During this process of homologous recombination, RAD51C is involved in the assembly of RAD51 foci. RAD51 forms a nucleoprotein filament on single-stranded DNA and catalyzes invasion of a homologous intact DNA template, forming a D-loop and priming DNA synthesis (Figure 5) [1, 9]. It is also thought that RAD51C is involved with the resolution of Holliday junction intermediates [1,4,9].

Homologous recombination is important in maintaining genome stability and preventing cancer. Because of this, *RAD51C* has been classified as a tumor suppressor gene due to its role in HR and cancer prevention [9]. Mutations in *RAD51C* have specifically been linked to breast and ovarian cancer and *RAD51C* is a designated breast/ovarian cancer susceptibility gene [6,9]. In fact, monoallelic mutations in

RAD51C can lead to a 2-3 fold increased risk of developing breast cancer and *RAD51C* germline mutations were present in 1.3% of families with both breast and ovarian cancer [7, 8].

The Relationship between RAD51C and FA

In 2010, a study was published in *Nature Genetics* that identified a new gene mutation resulting in a Fanconi-anemia like disorder [18]. This mutation was a homozygous missense mutation that led to an amino acid substitution within the RAD51C gene [5,18]. The RAD51C protein is known to be important in DNA damage repair, but had not previously been associated with Fanconi anemia (FA). This paper provided a step towards identifying previously unknown causes of FA. This is important because currently some FA patients have unknown causative mutations meaning that more mutations remain to be discovered [19].

The family discussed in the paper had a child with a bi-allelic missense mutation in RAD51C (R258H) [5,6,9]. This child displayed phenotypes characteristic of FA such as accumulation of DNA in the G2-M phase of the cell cycle, chromosome instability, sensitivity to crosslinking agents, and developmental abnormalities [9, 18]. The child did not, however, display hematological abnormalities or any cancers. This is unusual of downstream mutations which typically result in tumors and bone marrow failure at an early age [5, 19]. Therefore, the RAD51C mutation was classified as causing an FA-like disorder as opposed to full Fanconi anemia [19].

This 2010 study only found this RAD51C mutation in one family so it cannot be confidently stated that the RAD51C mutation causes FA generally. Until additional evidence of the relationship can be provided, RAD51C has provisionally been referred

to as FANCO [1,4,14,15]. To further clarify and expand upon the role of RAD51C in the FA pathway, my project focused on recreating the mutation in zebrafish and then analyzing the phenotype and gene expression patterns of the RAD51C mutants to provide additional tests of the hypothesis that RAD51C is a Fanconi anemia gene.

Research Outline

To understand how the *rad51c* mutation affects zebrafish, my research is broken into five main parts:

1. What is the gene expression pattern of *rad51c* in wild-type fish during early development?
2. What are the expression patterns of six hematopoietic genes in both mutant and wild-type zebrafish during early development?
3. How do *rad51c* mutants respond to DNA crosslinking agents?
4. Is there an increased prevalence of female-to-male sex reversal among *rad51c* mutants?
5. Do *rad51c* mutants exhibit reduced fertility characteristic of other characterized Fanconi mutants?

By answering these questions, we will be able to identify the role of *rad51c* in DNA repair and determine if the mutation results in characteristic FA symptoms.

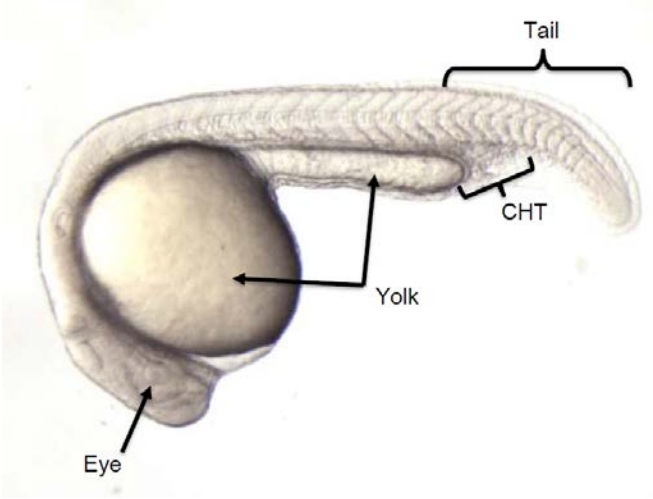


Figure 6: Anatomy of zebrafish embryo

This diagram illustrates the location of the eye, yolk, tail, and caudal hematopoietic tissue (labeled CHT). Picture accessed from http://sitn.hms.harvard.edu/wp-content/uploads/2014/02/KWR_pic3.png.

For all experiments, zebrafish (*Danio rerio*) were used as a model organism (Figure 6). This fish is a good model organisms for many reasons. First of all, it has been shown that the Fanconi anemia gene network is conserved from zebrafish to humans [16]. Zebrafish have an orthologous gene to the *RAD51C* gene found in humans as well as homologous genes to the rest of the FA network. This is important because we want the FA/BRCA pathway to be as similar to humans as possible. Zebrafish also make good model organisms because they have a short generation time, produce large numbers of transparent embryos, and share many developmental and physiological processes with humans [16].

The mutant zebrafish used for this research had a CRISPR-induced mutation in the *rad51c* gene located on chromosome 10. This mutant line was created by Sam Peterson, a former post-doctoral researcher in the Postlethwait laboratory. The mutation consists of a 16 bp deletion in exon 2 causing a frameshift mutation and an early stop codon, which would be expected to destroy the protein's function (Figure 7). The wild-

type zebrafish used in these experiments were AB strain.

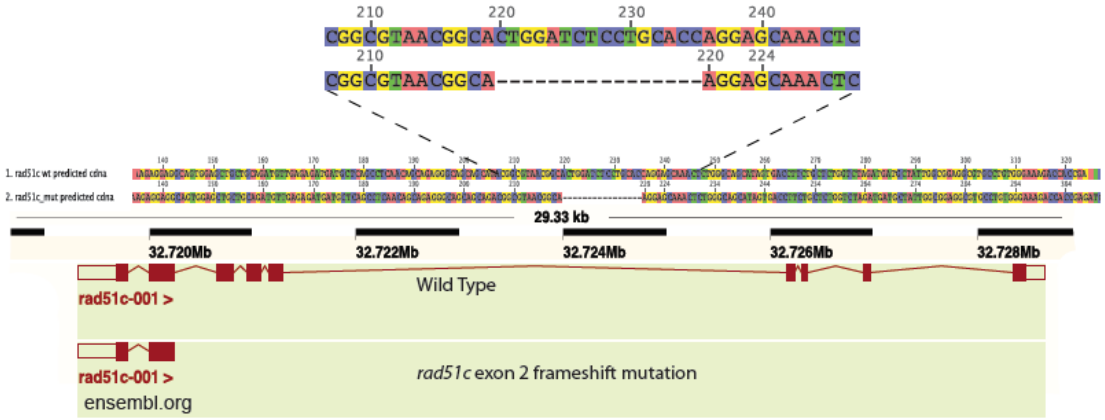


Figure 7: The mutation in *rad51c*

The *rad51c* mutation introduced in the zebrafish was a 16bp deletion causing a frameshift mutation.

Materials and Methods

Gene Expression

To determine the gene expression pattern of *rad51c*, we performed in-situ hybridization experiments to visualize where the gene is expressed. This was done through the following process:

Cloning and Sequencing

The first step was to amplify a fragment of the gene of interest using a polymerase chain reaction (PCR) and primers designed specifically for the gene in order to generate many copies of the gene. The gene fragment is amplified from zebrafish cDNA—DNA that is reverse transcribed from messenger RNA. This is done so that the fragment being amplified only contains the gene sequence because all of the introns have been spliced out. This allows the resulting probe to interact only with the mRNA, not the genomic DNA, allowing visualization of gene expression.

Once the gene had been amplified, it was mixed with a vector plasmid so that the gene could be incorporated into the plasmid (Figure 8). This plasmid had already been constructed to contain an Ampicillin resistance gene so that bacteria can grow even when exposed to Ampicillin. Next, 1 μL of the plasmid was inserted into 25μL of an *E. coli* bacteria culture and the bacteria were allowed to grow overnight at 37°C. Each time the bacteria replicate they replicate the gene of interest, hence the term "cloning".

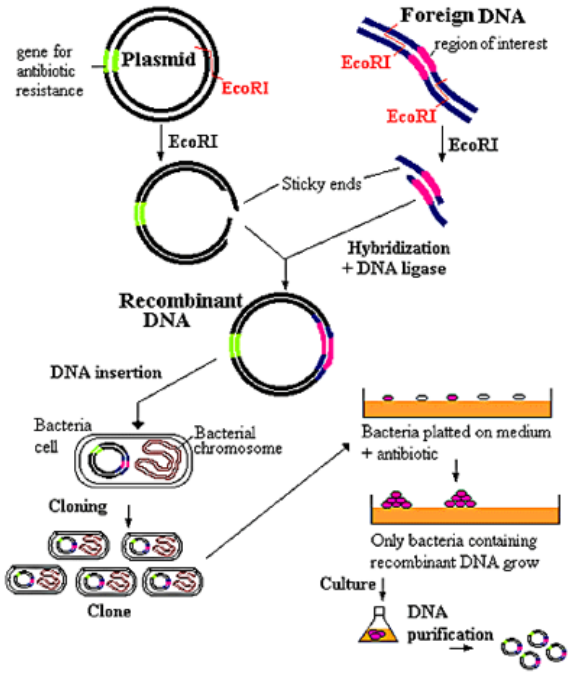


Figure 8: The cloning process

Image retrieved from http://andrus-dna-tech-project-2012.wikispaces.com/Xandria+Perman

The bacteria are grown on Ampicillin treated agar plates to ensure that only the bacteria with successfully inserted plasmids are growing—bacteria that do not contain the resistance gene will die. The next day, successfully grown colonies were picked, allowed to grow in isolation in LB+amp broth overnight, and then underwent a process

that extracts the plasmid. This process, known as plasmid preparation, involves the lysing of the bacterial cells and the purification of the plasmid. For this experiment, plasmid preparation was done using the reagents and protocol of the Zyppy Plasmid MiniPrep Kit. Once the plasmid was purified it was sent off for sequencing to verify the gene seqeunce and determine the direction in which the gene was inserted into the plasmid (3' to 5' vesus 5' to 3'). The samples were sequenced by Genewhiz using Sanger sequencing.

Making the Probe

When the sequencing results returned, the plasmid underwent PCR amplification so that a linear form of the gene was amplified. This was done by using forward and reverse M13 primers which are present in the plasmid at either end of the inserted gene so that only a linear copy of the gene is replicated. After this amplification, the PCR product was purified to separate out the gene using 25μL Diffinity RapidTip pipette tips. The linearized DNA then underwent transcription so that we obtained an RNA transcript. This was done by combing 5.5μL the gene with 2μL of 5x transcription buffer, 1μL of 10x nucleotide mix, 0.5μL RNase inhibitor, and1μL of the approiate T3 or T7 RNA polymerase. This is determined based of the orientation of the gene which is revealed in the sequencing results. The RNA was then purified and eluted using the Zymo RNA Clean & Concentrator-5 kit. This produces an RNA fragment that is able bind to mRNA in the fish, allowing us to visualize where the gene is expressed.

In-situ Hybridizations

The next step is to hybridize the probe to the messenger RNA in the zebrafish. To do this, fixed zebrafish embryos are first rehydrated with a series of methanol/PBT washes. Then the embryos are treated with proteinase K (10μg/mL in PBT) to increase the permeability of the membrane so that the probe can enter and bind. Following proteinase K treatment, the embryos are fixed in 4% PFA to prevent them from falling apart, then they are washed in PBT. After this they are washed in a solution of 50% formamide, 5x SSC, and 0.25% Tween-20, then they are put in hybridization solution and allowed to incubate at 68-70°C for two hours. After two hours, the embryos are put in hybridization solution containing the probe (0.5μL probe for 200 μL of hybridization solution) and are incubated with the probe overnight. The next day the embryos are washed in a series of mixtures of 50% formamide, 5x SSC, 0.25% Tween-20, and 2x SSC, 0.25% Tween-20. Then a 1:10,000 dilution of sheep digoxigenin-alkaline phosphatase Fab fragments is added. This antibody binds to the probe, incubating overnight at 4°C. From there, the embryos are washed in PBT, equilibrated in NTMT buffer (5M NaCl, 1M $MgCl_2$, 1M Tris pH 9.5, and 20% Tween-20), and then the dyes NBT and BCIP are added. These dyes stain the antibody which is bound to the probe, indicating the presence of the probe in the embryo and allowing visualization of where the probe is bound. The embryos are then imaged and analyzed to see in which tissues and at what developmental stage the gene of interest in expressed.

This process was performed for the *rad51c* gene for both *rad51c* mutant fish and wild-type fish at the developmental stages of 6hpf, 1dpf, 2dpf, 3dpf, 4dpf and 5dpf to see where the gene is expressed in the developing embryo and how the mutation affects

gene expression. Additionally, in situ hybridizations of the hematopoietic markers *gata1a, lcp1, ikzf1, lmo2, hbae,* and *spi1b* were performed in mutant fish at the developmental stages 1dpf, 2dpf, 3dpf, 4dpf, and 5pdf to see if the mutation affects hematopoietic development.

Genotyping

The in-situ hybridizations were performed on the fish of a *rad51c* heterozygous in-cross meaning that they had to be genotyped to determine if they were mutant, wild-type, or heterozygous fish. A cross of this type is expected to contain 25% mutants, 25% wildtypes, and 50% heterozygotes. To do so, the fish were put in 50 μL of lysis buffer and 10μL of 10mg/mL proteinase K. They were then heated at 55°C for 16 hours to breakdown the proteins and extract the DNA followed by 98°C for 10 minutes to denature the proteinase. From there the DNA undergoes the PCR protocol using the primers developed by Sam Peterson. The gene-specific primers for the wild-type *rad51c* gene were GCGTAACGGCACTGGATCTC/CTGAGTCTTTCCGACCCCAG and the primers for the mutated *rad51c* were GACGGCGTAACGGCAAGGAG/ CTGAGTCTTTCCGACCCCAG.

Sensitivity to DNA Crosslinking Agents

We exposed zebrafish embryos from a *rad51c* +/- in-cross to 1,2,3,4-Diepoxybutane (DEB) at 7 hours post fertilization. DEB is a genotoxic agent that induces DNA crosslinks. The fish were treated with either 0 ng/mL DEB, 175 ng/mL DEB, or 300 ng/mL DEB from 7-30 hours post fertilization. At 30 hours, the fish were washed 3 times in E2 fish water. They were then dechorionated and stained with 5

mg/mL acridine orange for 1 hour. Acridine orange binds to broken DNA, enabling the visualization of damaged DNA. After staining, fish were washed 3 times with E2 fish water, then anesthetized and imaged on a spinning disc confocal microscope.

Once the DEB-treated fish were imaged, they were genotyped as previously described. Images were loaded into ImageJ and the threshold tool was used to select acridine-orange stained cells. Then the area of stained cells in the tail, from the end of the yolk to the tip of the tail, was quantified with the measurement tool.

Sex Ratios

To discover whether *rad51c* mutations lead to a Fanconi phenotype in zebrafish, we performed a sex ratio analysis of a *rad51c+/-; tp53+/-* cross. The phenotypic sex of each fish was verified by visual observation of external morphological characteristics. Female zebrafish have a larger rounder belly and a prominent cloaca while male zebrafish have a more streamlined physique and a bright orange anal fin (Figure 9). These fish were separated, counted, and genotyped using the PCR protocol mentioned above. DNA was extracted by adding 50μL of 50 mM NaOH to a fin clipping and heating for 20 minutes at 98°C.

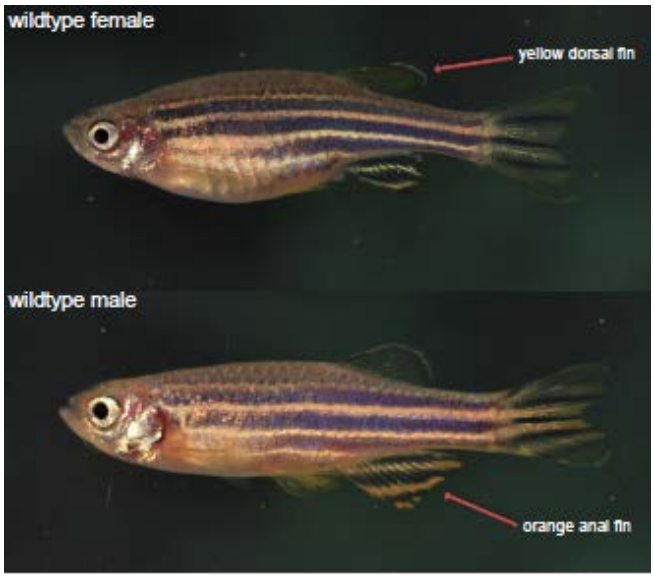


Figure 9: Male and female zebrafish phenotype.

Photos by Thomas Desvignes of the Postlethwait Laboratory.

Fertility Assays

To examine the fertility of zebrafish with a *rad51c* mutation, fertility assays were performed on a *rad51c; tp53* heterozygous in-cross fish that had previously been genotyped. Each fish being assayed was crossed with three wild-type fish of the opposite sex. Three fish of the opposite sex were used to minimize the chance of a fish not laying eggs and to increase the likelihood of a good spawn. The embryos were then manually counted and classified as either fertilized or unfertilized. In some instances, fertilized eggs were allowed to develop to 24 hours post fertilization at which point they were visually examined for developmental abnormalities.

Results

Gene Expression

The in-situ assays for gene expression results show that *rad51c* is ubiquitously expressed in the 6hpf wild-type fish. Among fish at 1dpf-5dpf, *rad51c* is broadly

expressed, with higher concentration seen in rapidly dividing cells in the head and brain (Figure 11). High expression is also seen in the heart during early development as well as the jaw at 4dpf.

The results of the hybridizations on mutant fish show that expression of the six hematopoietic genes is normal. I conclude that the *rad51c* mutation does not affect hematopoietic development it zebrafish embryos (Appendix 2-Appendix 7). While it may appear that the expression of *gata1a* differs between wild-type and mutant fish for 1dpf and 2dpf fish, this may be due to individual variation in expression (Figure 10). Using ImageJ to measure the area of expression in the caudal hematopoietic tissue between wild-type and mutant results in the p value of 0.366 suggesting that this variation is not a significant difference.

Figure 10: Variation in *gata1a* expression among 1dpf fish.

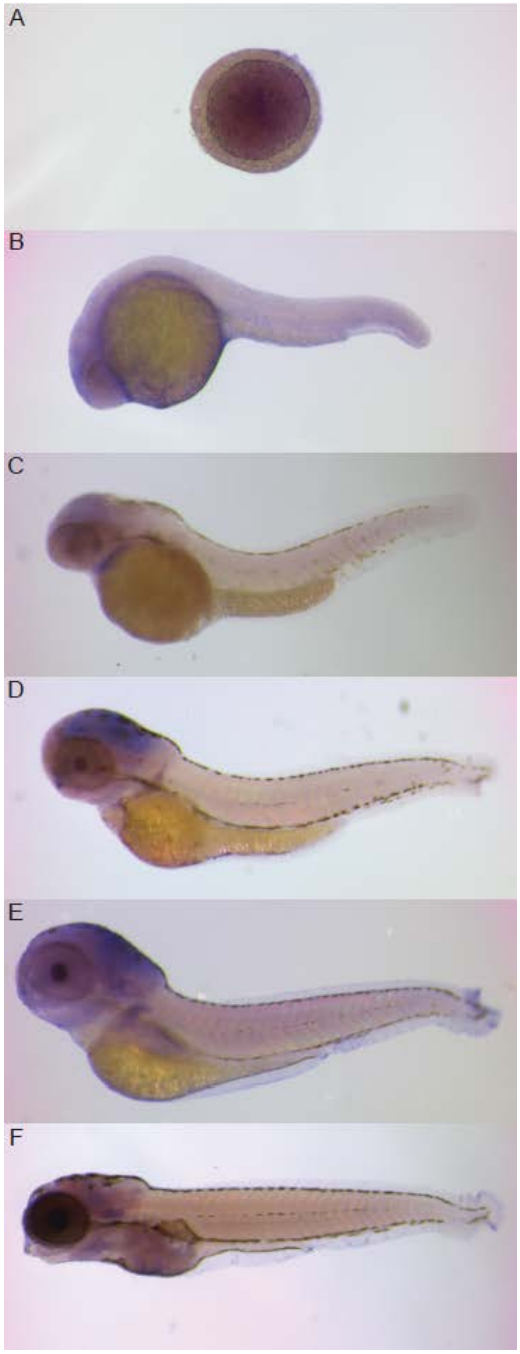


Figure 11: Expression pattern of *rad51c* in wild-type zebrafish from 6hpf-5dpf

A) 6hpf, *rad51c* gene is ubiquitously expressed B) 24hpf, expression is found mostly in the head and concentrated in the brain C) 2dpf, expression is found in the brain and heart D) 3dpf, expression is concentrated in the brain E) 4dpf, expression is concentrated in the head, including the brain and jaw F) 5dpf, expression is found in the head, including the brain, as well as the heart.

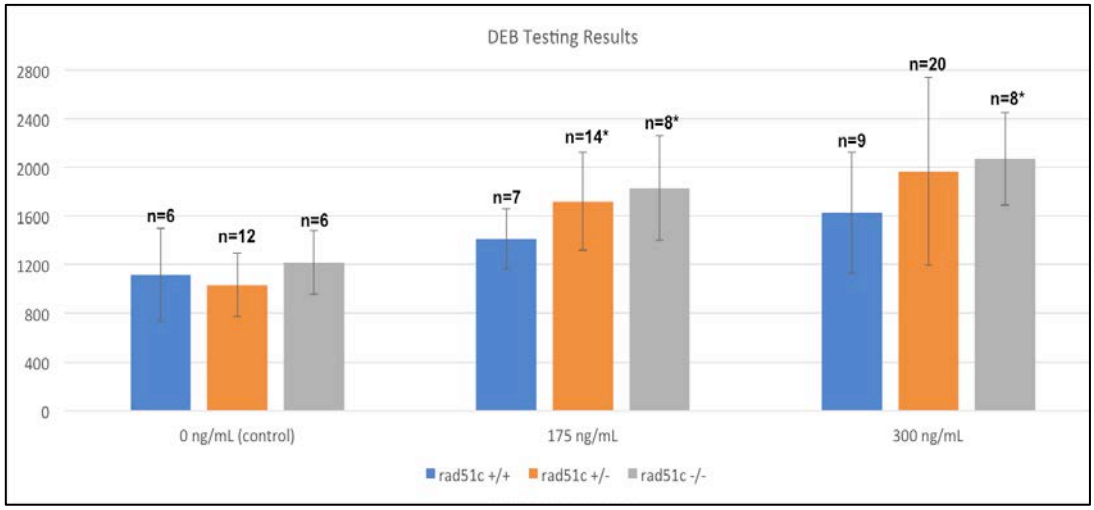


Figure 12: Results of DEB testing

There is a significant increase in DNA damage among heterozygous and mutant fish when treated with 175 ng/mL of DEB and among mutant fish when treated with 300 ng/mL DEB (*=p-value < 0.05).

DNA Crosslinking

The DEB testing results showed that there is no statistically significant difference in the amount of DNA damage between the genotypes in the control group (Figure 12). Statistical significance was determined using a one-tailed t-test for two samples of unequal variance. Upon exposure to 175 ng/mL DEB, both the heterozygous and mutant zebrafish displayed a significant increase in the amount of DNA damage compared to wild-type fish (p=0.021 and 0.018, respectively). After treatment with 300 ng/mL DEB, mutant fish show a statistically significant increase in DNA damage compares to wildtype with a p-value of 0.028. Heterozygous fish also show an increase in DNA damage compared to wildtypes but this difference is not quite significant (p=0.085). Although mutant fish appeared to show a minor stepwise increase in the amount of damage compared to heterozygous fish, this increase did not appear to be

significant (p=0.17 at 175ng/mL and p=0.32 at 300ng/mL). This result could possibly be due to a small sample size.

Sex Ratios

The data show that *rad51c* mutants develop only as males (n=42). This holds true for both *rad51c -/-; tp53 +/+* (n=14) and *rad51c -/-; tp53 +/-* (n=28). To test whether programmed cell death is required for *rad51c* mutants to experience sex reversal, I made double mutants for *rad51c* and *tp53*, a gene required for cells to undergo apoptosis. Results showed that *rad51c -/-; tp53-/-* double-mutants developed as both males and females in approximately equal numbers, eight females and nine males (Figure 13). I conclude that *rad51c* mutants undergo sex reversal due to programmed cell death induced by *tp53* and that *rad51c* is required to prevent female-to-male sex reversal.

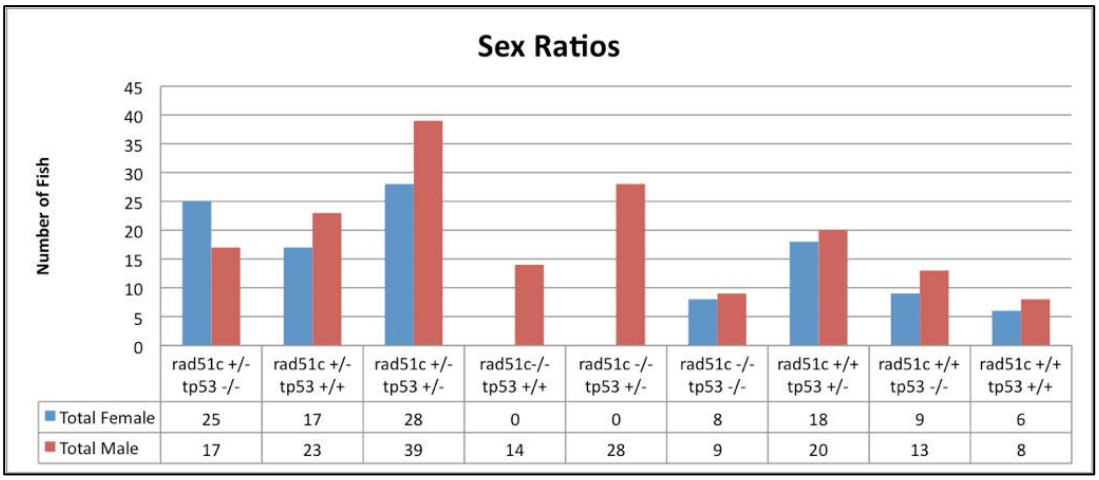


Figure 13: Sex ratio results

These sex ratios show that *rad51c* mutants develop only as males and *rad51c; tp53* double mutants develop as both males and females.

Fertility Assays

The fertility assays were performed on a total of 46 fish. The results of the assays show that *rad51c* mutant female zebrafish have mostly sterile eggs that are unable to be fertilized (Figure 14). Wild-type (n=4) and heterozygous (n=7) fish had 84% and 72% of their eggs fertilized on average, respectively, In contrast, *rad51c* mutant females (n=5) only had roughly 5% of their eggs fertilized, on average. In fact, 40% of the mutant females tested produced no fertile eggs. Mutant females ranged from having 0% of their eggs fertilized to 9.3%. Also, when an egg was able to be fertilized, the embryo developed abnormally with asymmetrical cellular development (Figure 15). None of the fertilized embryos that were observed survived to 24hpf.

Fertility assays showed that *rad51c* mutant male zebrafish have severely reduced fertility compared to wild types. On average, wild-type (n= 6) and heterozygous (n=6) males were able to successfully fertilize 62% and 56% of laid eggs, respectively. Conversely, *rad51c -/-; tp53 -/-* males (n=7) only successfully fertilized 8.7% of eggs and *rad51c -/-; tp53 +/+* males (n=11) only fertilized 2.5% of eggs. However, when a mutant male was able to successfully fertilize an egg, the embryo was able to develop normally and observations show that the fish successfully survive (Figure 16).

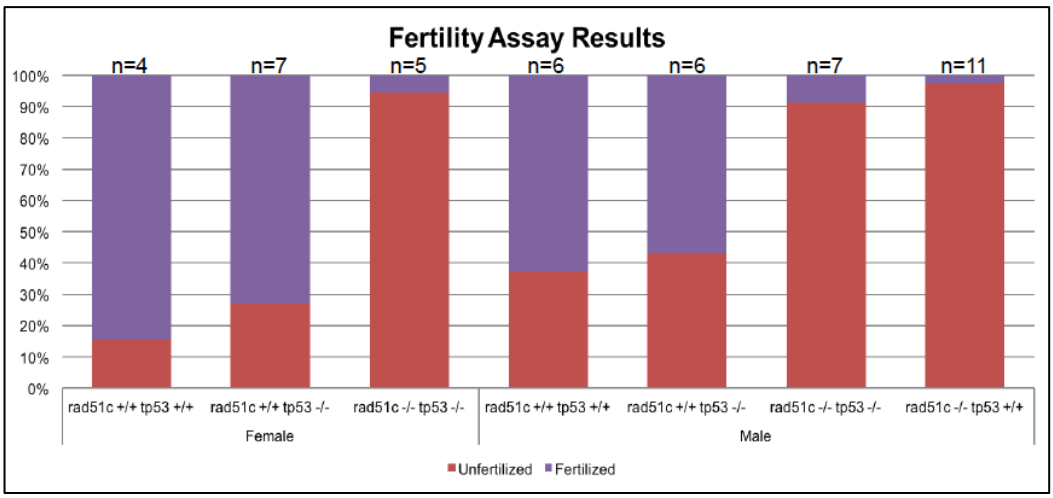


Figure 14: Fertility assay results

These results show severely reduced fertility among *rad51c* mutants.

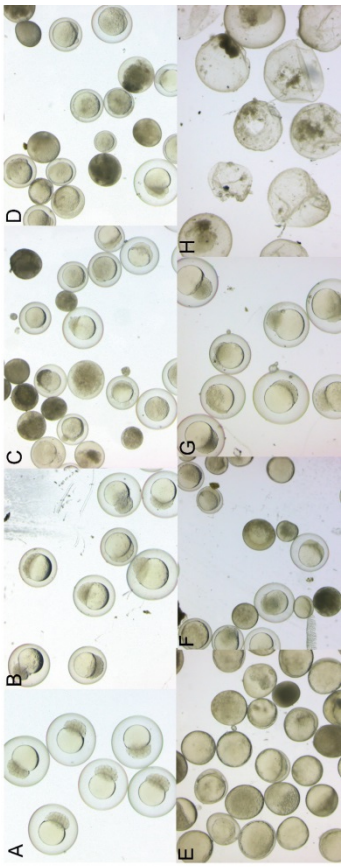


Figure 15: Embryos of female *rad51c* mutant zebrafish

A) fertilized wild-type embryos B) *rad51c; tp53* double mutant embryos fertilized by wild-type fish showing abnormal, asymmetrical development C-D) *rad51c; tp53* eggs showing large proportion of sterile eggs and eggs of varying sizes E) *rad51c; tp53* sterile eggs with small chorion F-H) *rad51c; tp53* eggs that are mostly sterile, G shows the few fertilized eggs from the same female in F, H shows the fertilized eggs from panel G at 1dpf

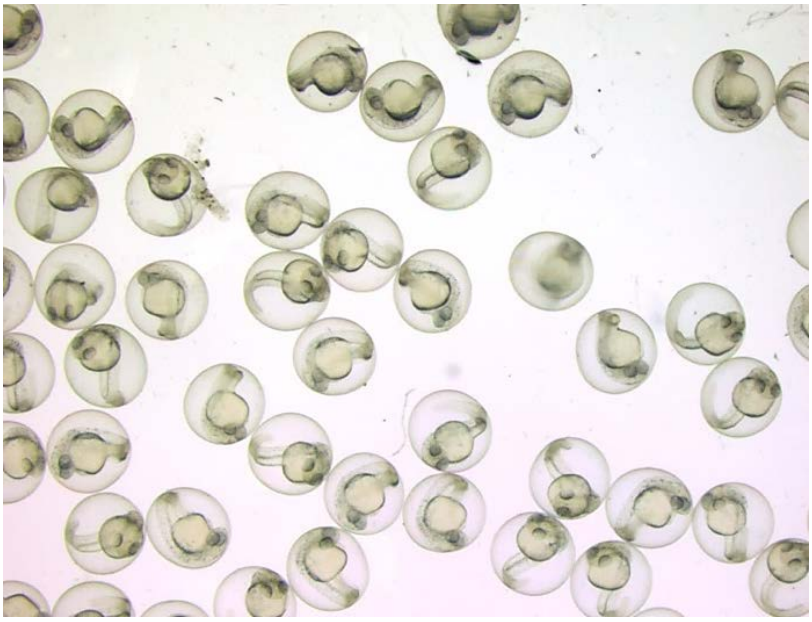


Figure 16: Embryos of male *rad51c* mutant at 1dpf

This figures shows that successfully fertilized embryos of a wild-type female and *rad51c* mutant male demonstrate normal development.

Discussion

Gene Expression

In the wild-type fish, the ubiquitous expression of *rad51c* in rapidly dividing tissues such as the brain is consistent with what would be expected of a DNA repair gene. This is because tissues that are rapidly dividing are simultaneously rapidly replicating DNA to put in new cells so the DNA is more prone to damage and therefore the DNA repair pathway is more active. It has been found that *fanc* genes are strongly expressed in proliferating neural cells because of the DNA damage associated with rapid cell division [17].

Of the six hematopoietic genes tested, all were normally expressed in the *rad51c* mutant fish. This suggests that hematopoietic development is unaffected by the

mutation. Normal expression of *hbae* indicates hemoglobin development in not affected (Appendix 2). *Gata1a* and *lmo2,* which are genes involved in erythropoiesis, are also normally expressed in the mutants suggesting normal red blood cell development (Appendix 3 and Appendix 6). Furthermore, *lcp1*and *ikzf1* are also normally expressed indicating normal lymphocyte development (Appendix 5 and Appendix 4). Lastly, *spib*1, a gene involved in myeloid development is also normally expressed (Appendix 7). By comparing the development of these different processes of hematopoiesis, it is observed that *rad51c* mutations do not interfere with blood cell development. This observation of no hematological abnormalities is uncharacteristic of downstream FA mutations, but does help to explain why *RAD51C* mutations do not seem to result in bone marrow failure in humans.

DNA Crosslinking

As previously discussed, a characteristic of FA patients is sensitivity to DNA crosslinking agents. The hypothesis that *rad51c* is a member of the FA/BRCA pathway and is a causative gene of Fanconi anemia predicts that *rad51c* mutants should exhibit an increased sensitivity to the crosslinking agent DEB. Upon DEB treatment, both the mutant and heterozygous fish did exhibit an increased sensitivity to DEB resulting in increased apoptosis. This is because when *rad51c* is mutated and the protein is unable to function properly, the FA/BRCA repair pathway is hindered. This block inhibits the cells' ability to cope with the increased crosslinking that results from the DEB treatment and causes the cell to undergo apoptosis. These results support the characteristic Fanconi phenotype of increased sensitivity to crosslinking agents, supporting the role of *rad51c* as a Fanconi anemia gene.

Sex Ratios

One way of further confirming the connection between *rad51c* mutations and Fanconi anemia is by analyzing the sex ratios of *rad51c; tp53* double mutants. This is because in both humans and zebrafish one of the characteristics of FA is hypogonadism and reduced fertility [10]. Interestingly, zebrafish initially develop as hermaphrodites because juveniles develop gonads with immature oocytes regardless of their adult sex [10]. In homozygous FA mutants, however, the fish develop as males due to death of oocytes and hence undergo female-to-male sex reversal [10].

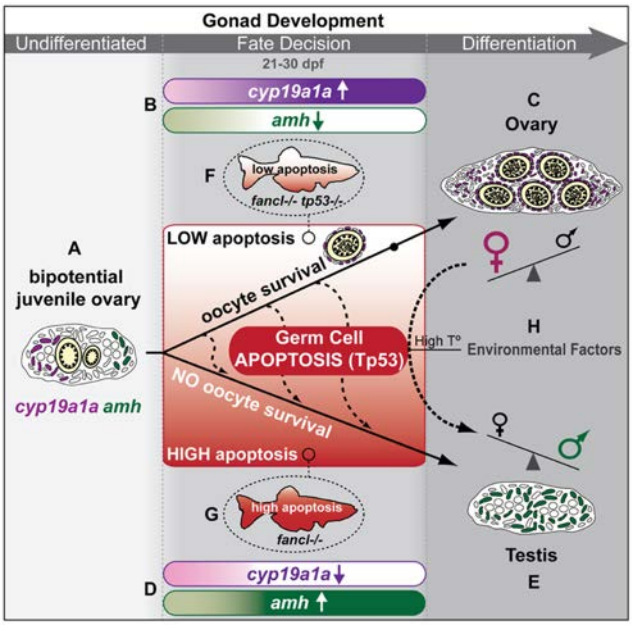


Figure 17: Pathway of female-to-male sex reversal in zebrafish

This diagram shows how mutating *tp53* reduces apoptosis, allowing ovaries to persist and fish to develop as females (Rodríguez-Marí A, Cañestro C, BreMiller RA, Nguyen-Johnson A, Asakawa K, Kawakami K, et al., 2010, PLoS Genet 6(7): e1001034.).

This sex reversal occurs because the oocytes undergo meiosis and develop DSBs that cannot be repaired due to the mutation in the DNA repair pathway. Because of this DNA damage, the oocytes are unable to progress through meiosis and experience

increased germ cell apoptosis and compromised oocyte cell survival (Figure 17). This absence of oocytes causes the gonads to masculinize and become testes [10].

The sex ratio results show that this sex reversal phenotype can be rescued in *rad51c; tp53* double mutants [10]. *Tp53* (tumor protein p53) is a protein that induces apoptosis. Therefore, a mutation in this protein rescues the sex-reversal phenotype by reducing germ cell apoptosis, therefore allowing oocytes to persist and the fish to develop as females [10].

That *rad51c* mutants exhibit female-to-male sex reversal is consistent with all other characterized Fanconi anemia mutants, therefore supporting *rad51c* as a Fanconi gene.

Fertility Assays

Zebrafish with FA mutations exhibit various levels of fertility. Zebrafish with mutations in the FA core complex are fertile and those with mutations in the downstream *brca2* gene are sterile [10, 11]. The Postlethwait lab has shown that *rad51c* mutants fall in the middle of the spectrum and exhibit reduced fertility. To confirm this reduced fertility phenotype, fertility assays were performed on *rad51c; tp53* fish in order to determine the fertility level of the mutant fish and to examine if *tp53* is able to rescue fertility levels.

Results confirm that the *rad51c* mutation does lead to severely reduced fertility in zebrafish. This result represents an intermediate phenotype between the core complex mutants, which are fertile, and the *brca2* mutants which are sterile. It does not appear that knocking out *tp53* is able to rescue fertility.

The embryos of female mutants are not able to develop because the embryonic development of zebrafish relies on maternally deposited gene products. These products are generated during oogenesis and supplied to the egg, but as observed in the sex ratios, the female *rad51c* mutants have disrupted oogenesis and are creating unviable eggs. Because these eggs are lacking essential genetic material, the development of the embryos is unsuccessful. Conversely, although the zygotes of the male mutant have the same genotype, they are developing using the genetic information supplied by the wild-type mother so they can develop normally.

Concluding Remarks and Significance of Research

Future directions include analyzing histological sections of the gonads of double mutant females and *rad51c-/-; tp53-/-* and *rad51c-/-; tp53+/+* males to determine the anatomical causes underlying the reduced fertility phenotype. Possible mechanisms of reduced fertility in males include impaired spermatogenesis resulting in either no sperm produced or reduced sperm count. Other possibilities include failure to court females or failure to release sperm. Analyses of the testes is required for more evidence of reduced fertility.

The FA/BRCA pathway is a crucial pathway in repairing DNA double-strand breaks. DSBs are dangerous because they are a source of genome instability that can result in various cancers. For this reason, having a better understanding of the DNA damage repair process can provide insight on both the origins of and therapies for cancers. Specifically, by understanding which proteins are involved in the DNA repair pathway, we will be able to better identify risk factors for various cancer, leading to earlier detection and improved prevention [9]. Furthermore, identifying additional

mutations that result in Fanconi anemia will help to identify more causes of the disease allowing for better identification. By better understanding the RAD51C protein and its relation to human health, we are taking steps towards better prevention, identification and therapies for FA and cancer patients.

Glossary

Apoptosis: Process of programmed cell death

Autosomal: Pertaining to a chromosome that is not a sex chromosome

Biallelic: Of, relating to, or affecting both alleles of a gene

Cisplatin: A chemotherapy drug which is used to treat various cancers

Congenital: Existing at or before birth regardless of cause

Endogenous: Produced within or caused by factors within the organism

Exogenous: Originating or produced from outside the organism

Genotoxic: Damaging to DNA

Germline mutation: Any detectable and heritable variation in the lineage of germ cells. Mutations in these cells are transmitted to offspring, while, on the other hand, those in somatic cells are not. A germline mutation gives rise to a constitutional mutation in the offspring, that is, a mutation that is present in virtually every cell.

Gametogenesis: Biological process by which diploid or haploid precursor cells undergo cell division and differentiation to form mature haploid gamete

Heterogeneous: Diverse in character or content

Holliday junction: Cross-shaped structure that forms during the process of genetic recombination, when two double-stranded DNA molecules become separated into four strands in order to exchange segments of genetic information.

Homologous: A gene related to a second gene by descent from a common ancestral DNA sequence. The term, homolog, may apply to the relationship between genes separated by the event of speciation (see ortholog) or to the relationship betwen genes separated by the event of genetic duplication (see paralog).

In situ hybridization: A type of hybridization that uses a labeled complementary DNA, RNA or modified nucleic acids strand (i.e., probe) to localize a specific DNA or RNA sequence in a portion or section of tissue (in situ)

Lysing: A process in which a cell is broken down or destroyed

Meiotic recombination: The production of offspring with combinations of traits that differ from those found in either parent

Monoallelic: When only one allele of a gene is actively transcribed

Orthologous: Orthologs are genes in different species that evolved from a common ancestral gene by speciation. Normally, orthologs retain the same function in the course of evolution.

Paralog: A gene that is related to another gene in the same organism by descent from a single ancestral gene that was duplicated and that may have a different DNA sequence and biological function.

Plasmid: A plasmid is a small DNA molecule within a cell that is physically separated from a chromosomal DNA and can replicate independently

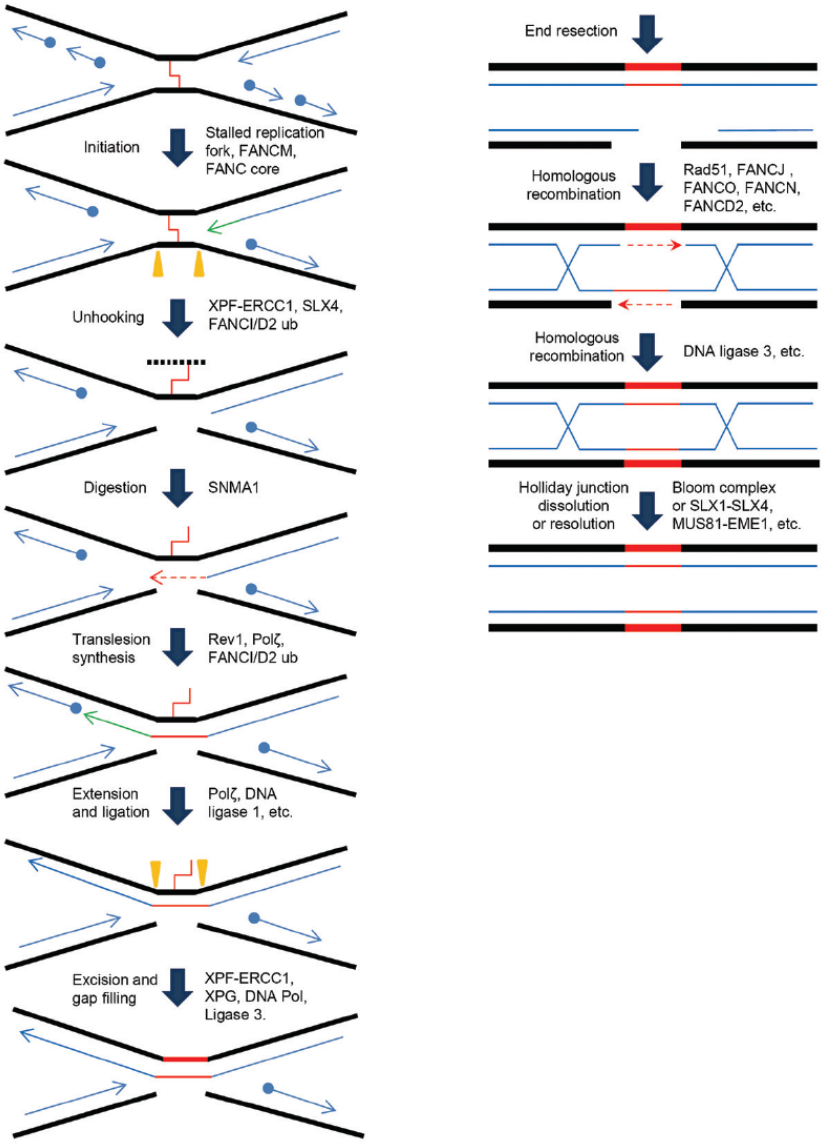
Polymerase chain reaction: A technology in molecular biology used to amplify a single copy or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence.

Primers: A primer is a short strand of RNA or DNA (generally about 18-22 bases) that serves as a starting point for DNA synthesis

Recessive: The recessive trait may be expressed when the recessive genes are in homozygous condition or when the dominant gene is not present. That happens when an organism inherits a pair of recessive genes from its parents.

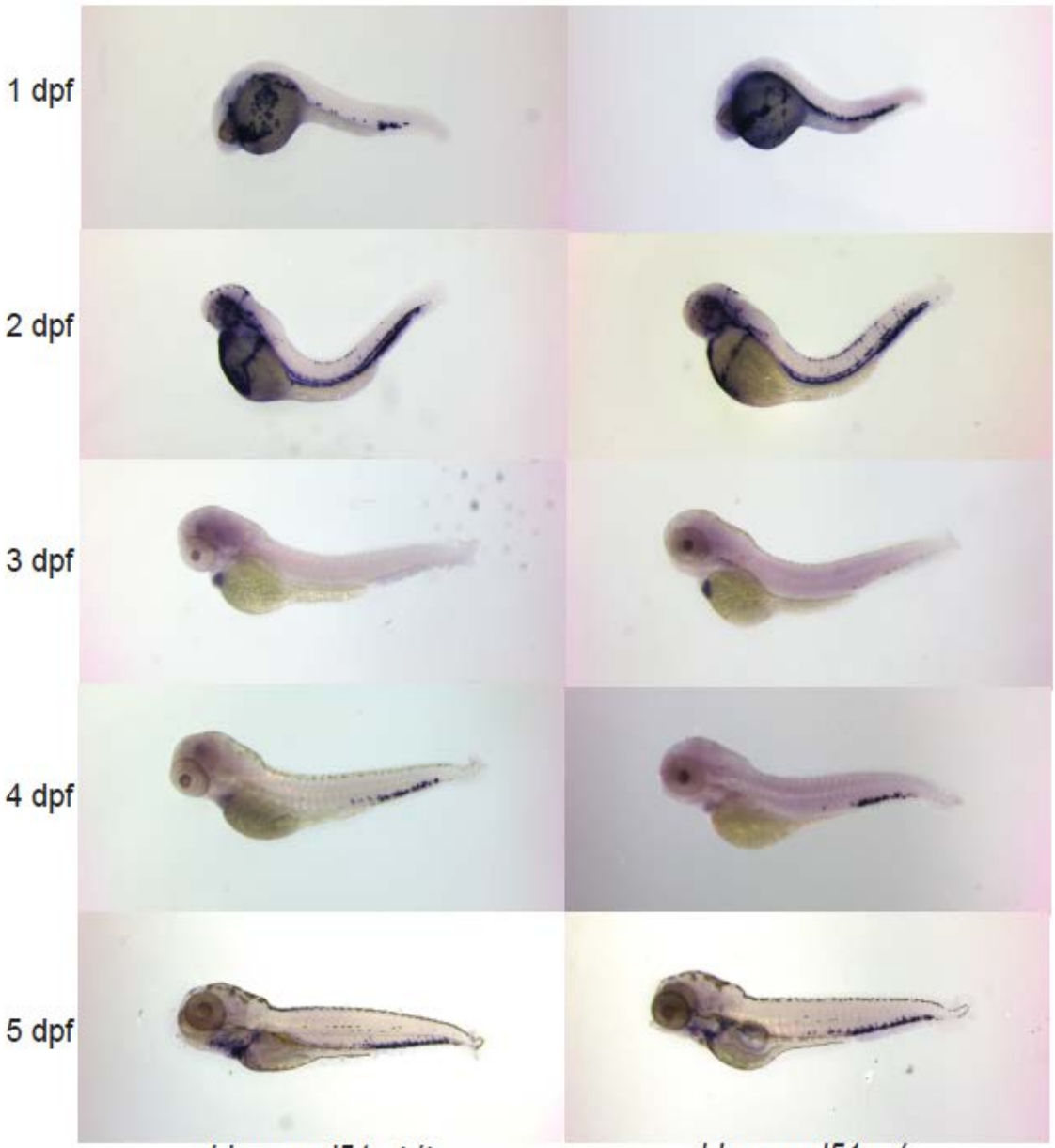
X-linked: Transmitted by genes on the X chromosome, a sex chromosome.

Appendix 1

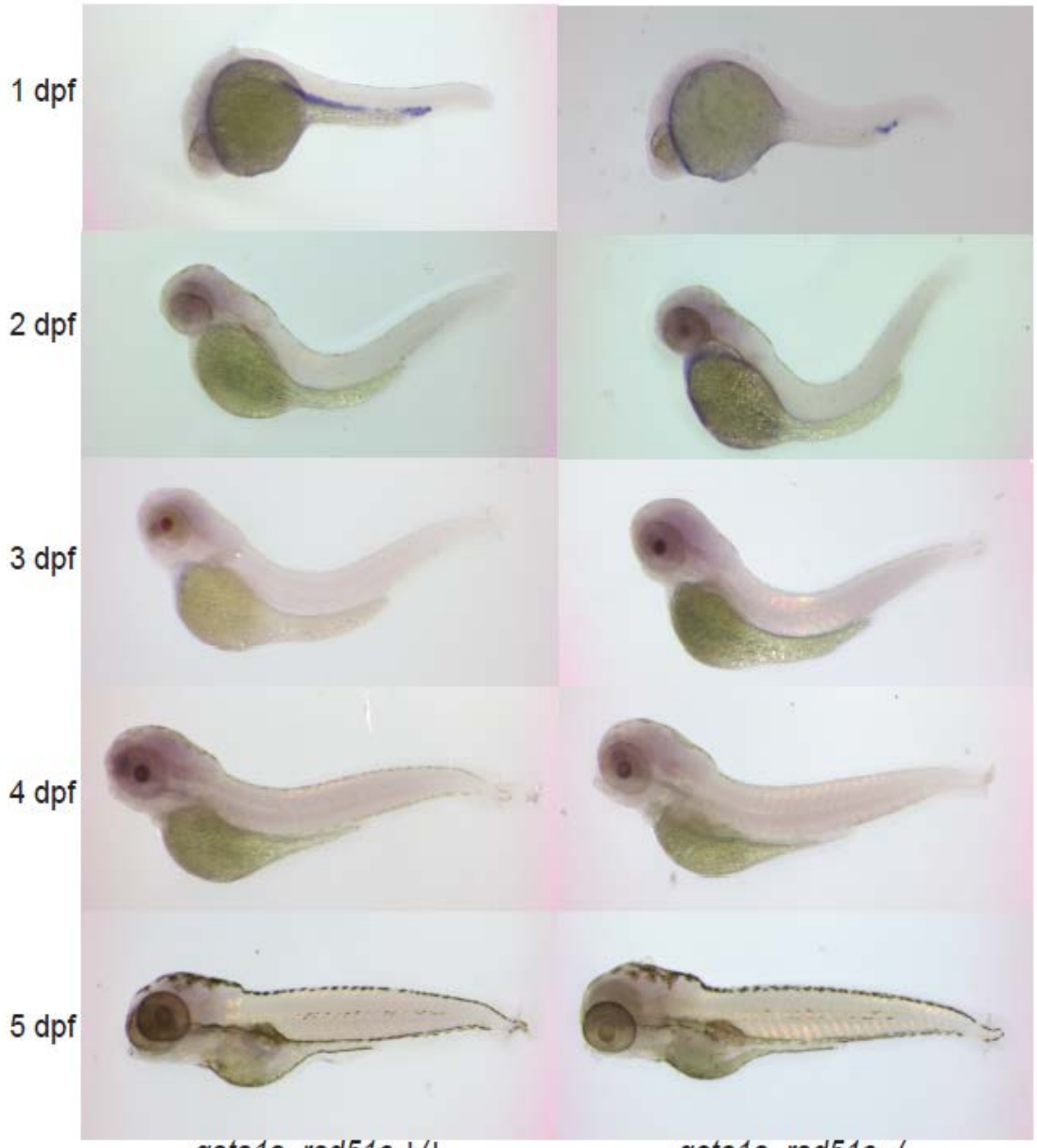


This diagram outlines the process of ICL repair via homologous recombination. First the ICL is detected by the FA core complex. Then nucleotide excision repair endonuclease (NER) unhooks the ICL. The FA complex recruits translesion synthesis, the crosslink is cleaved and one strand is repaired. Now homologous recombination occurs to repair the double strand break (right column). The 3' ends of the DNA are exposed and RAD51C and other HR proteins are recruited. Strand invasion occurs and DNA is synthesized. The resulting Holliday junctions are resolved and the DSBs is repaired.

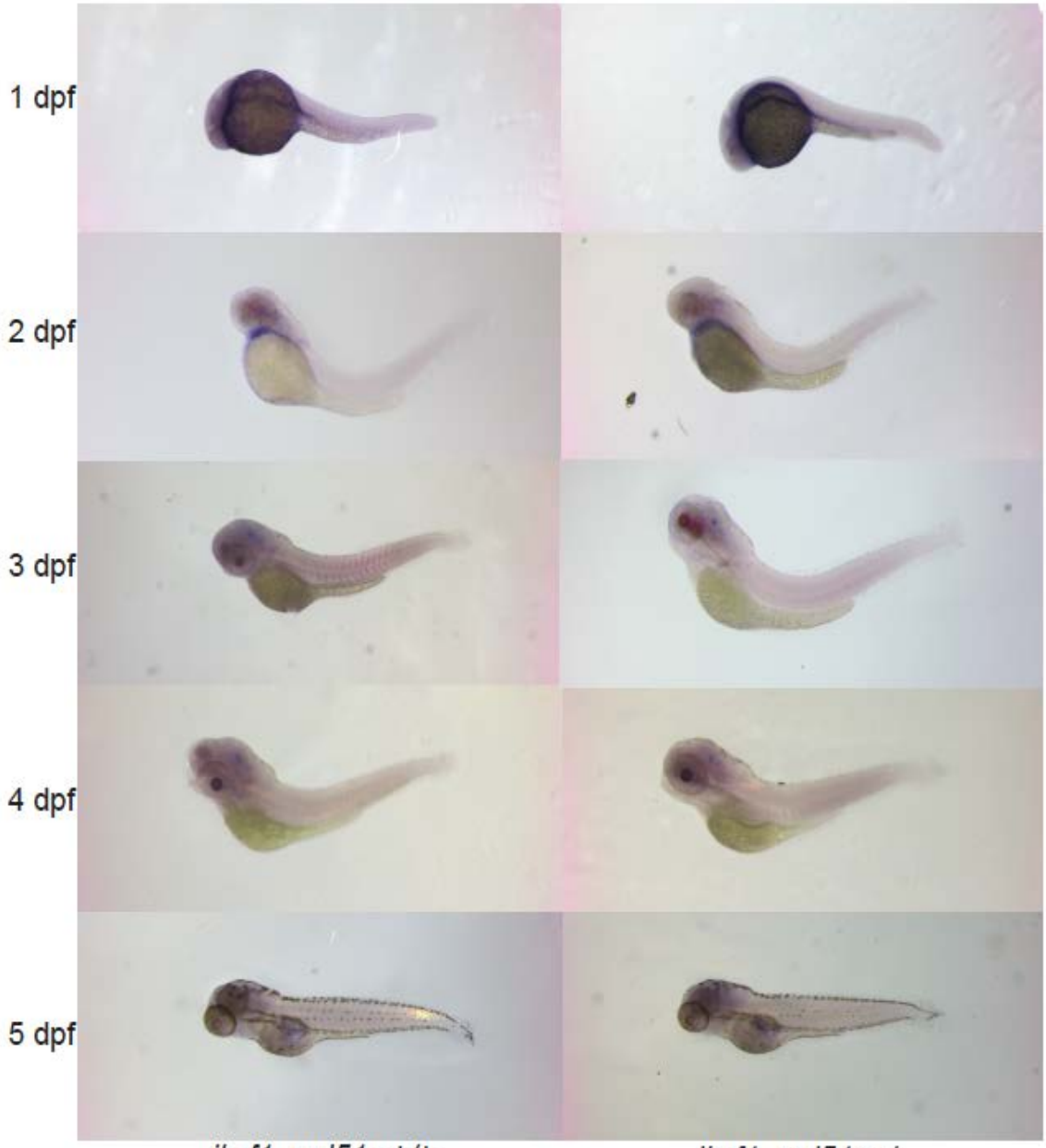
Appendix 2



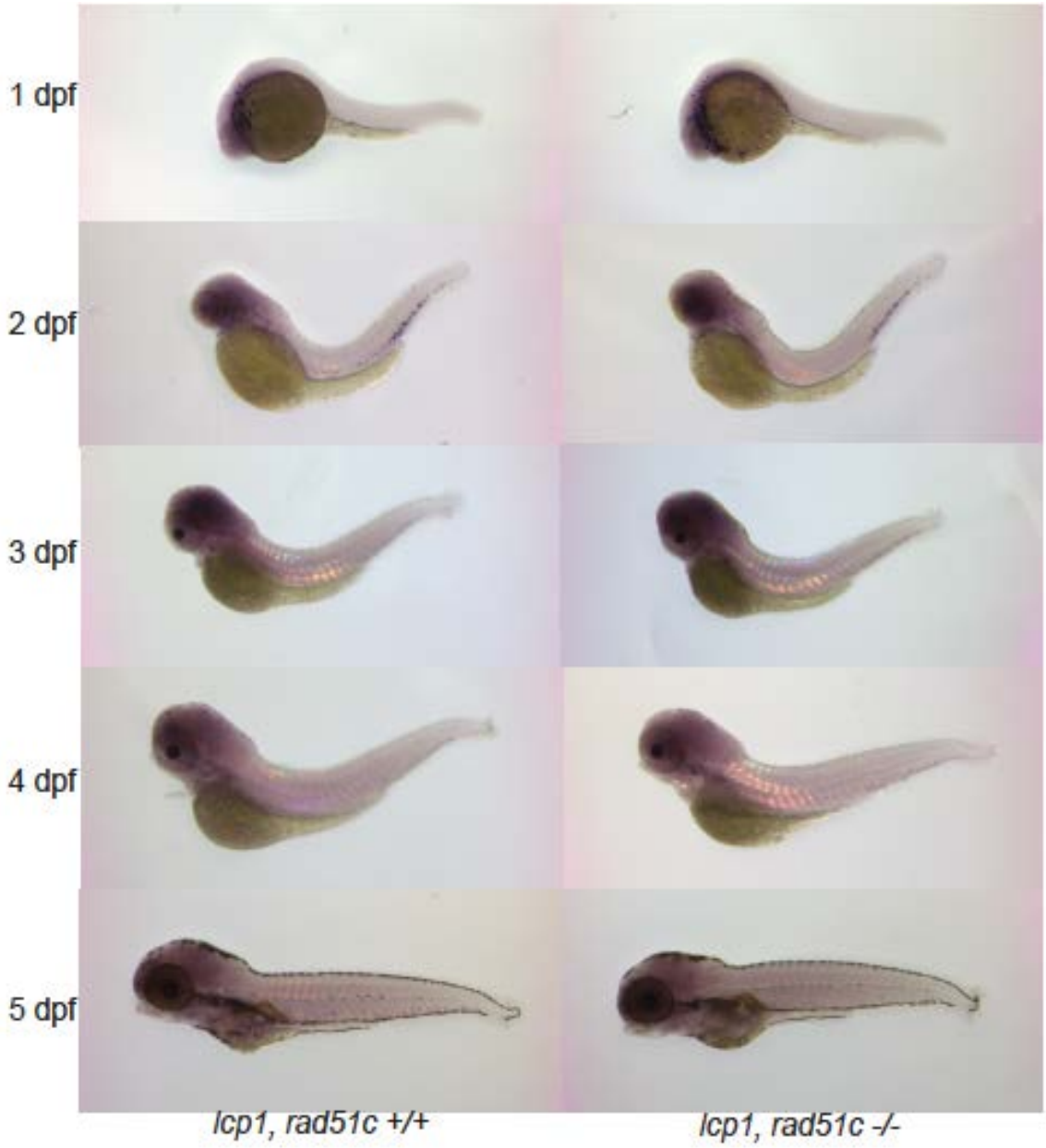
Appendix 3



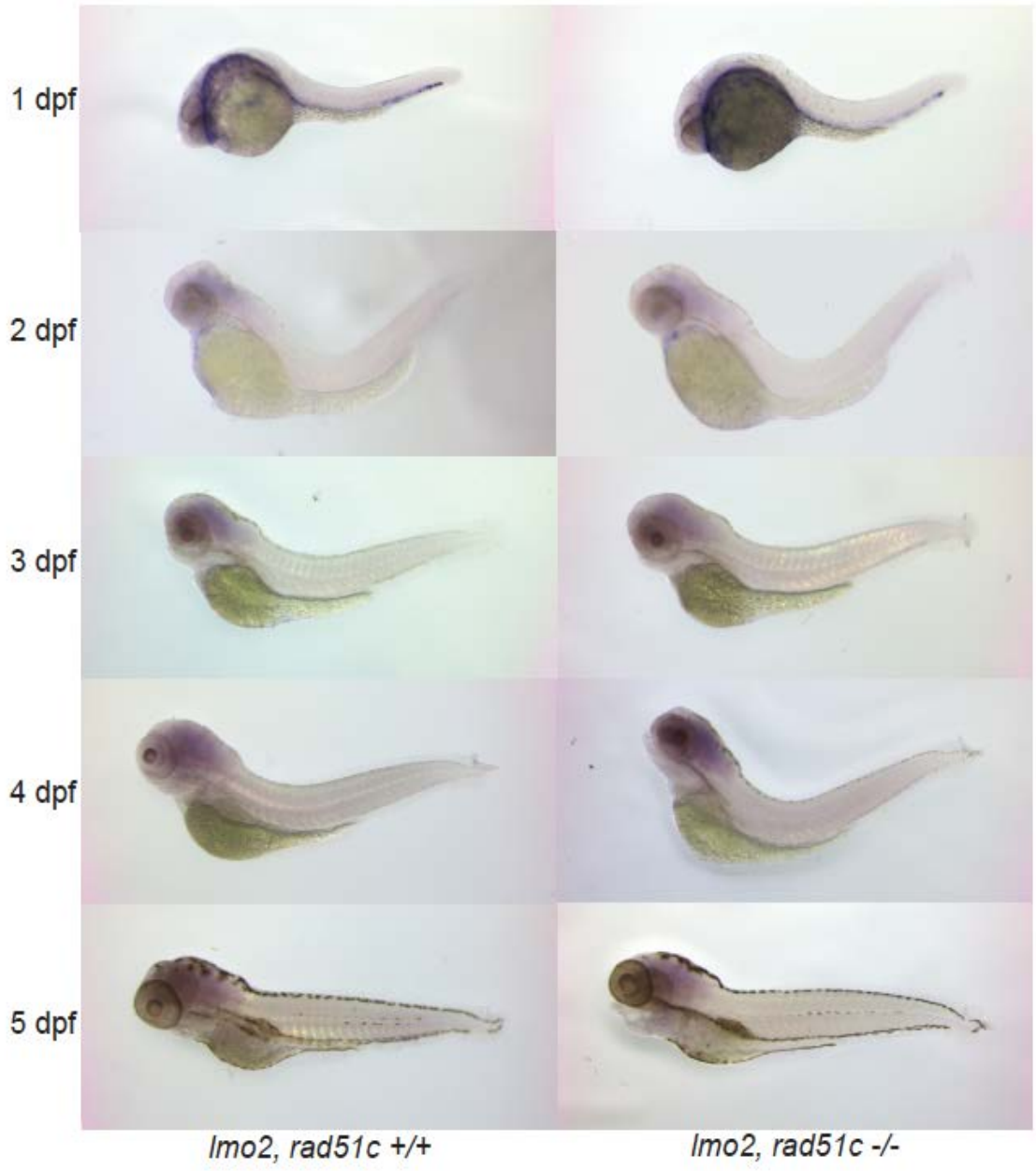
Appendix 4



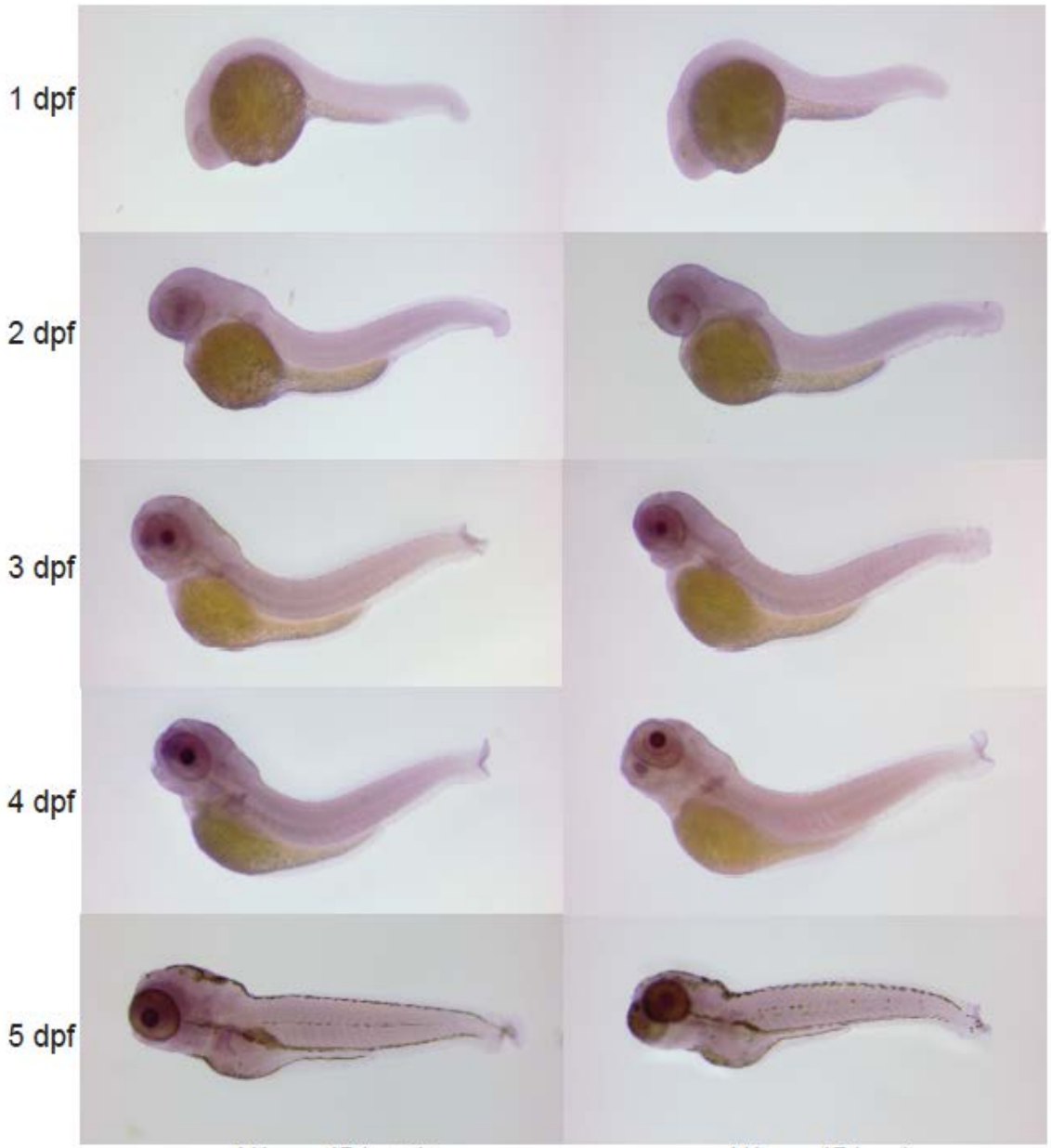
Appendix 5



Appendix 6



Appendix 7



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